

REMARKS

Reconsideration and allowance are respectfully requested.

Claims 19-24, 27-31, and 33-37 are now pending, with Claim 19 being the sole independent claim. Claim 38 is canceled without prejudice or disclaimer. Claims 19, 27, 31, and 33-35 have been amended. The specification has been amended to reflect the new numbering of the figures. No new matter is believed to have been added.

Turning now to the Office Action mailed April 10, 2003:

Regarding the Sequence Listing, Applicants submit that nucleotides 1-1329 (stop codon at 1330-1332) of SEQ ID NO:13 encode a polypeptide of amino acids 1-443 of SEQ ID NO:14.

Regarding the drawings, Applicants request entry of the attached amended drawings, as described above.

Regarding the Section 112, 2d paragraph rejections, Applicants have amended Claims 27, 31, and 33-35, as suggested. Withdrawal of the Section 112, 2d paragraph rejections is respectfully requested.

Regarding the Section 112, 1st paragraph (written description) rejection, Applicants respectfully traverse.

First, Applicants submit that the specification discloses to one of ordinary skill in the art a representative number of polynucleotides encoding polypeptide sequences having plant lecithin:cholesterol acyltransferase activity with at least 80% sequence identity to SEQ ID NO:14, and not just a single polynucleotide encoding SEQ ID NO:14.

The specification at page 6, lines 2-14, discloses alterations in nucleotide sequence that are not expected to alter functionality, such as alterations that produce a chemically equivalent amino acid at a given site or alterations in the N- or C-terminal portions. Thus, from the foregoing, the skilled artisan would immediately understand the specification to disclose a representative number of polynucleotide sequences, having different nucleotide substitutions, that encode polypeptides having plant lecithin:cholesterol acyltransferase activity but that vary (within 80% sequence identity) of SEQ ID NO:14.

Second, under the PTO Written Description Guidelines, the written description requirement is met with disclosure of “functional characteristics when coupled with a known or disclosed correlation between function and structure.” Guidelines, 66 Fed. Reg. at 1106.

Applicants submit that there is a known correlation between the plant lecithin:cholesterol acyltransferase activity disclosed and claimed in the instant specification and plant lecithin:cholesterol acyltransferase (and corresponding nucleic acid) structure.

Rogne et al., *Biochem. Biophys. Res. Comm* (1987) 148:161-169 (previously submitted; copy attached for convenience), discussed in the instant specification at page 3, line 37 to page 4, line 4, discloses a sequence motif that is conserved in mammalian lecithin:cholesterol acyltransferases. Within this motif the active site serine can be found, which appears to be conserved in all serine lipases analyzed to date. Moreover, segments predicted to be in alpha-helical conformation are shown in Figure 2 of Rogne et al.

Attached herewith on Appendix A is a comparison of SEQ ID NO:14 with the amino acid sequence disclosed by Rogne et al. As shown on Appendix A, the sequence of the invention possesses all but one of the residues within this distinctive, highly conserved motif and the active site residue (boxed region of the alignment on Appendix A). Furthermore, on Appendix A, other conserved amino acids are identified by asterisks, and the segments predicted to be in alpha-helical conformation are underlined.

Applicants submit that the combination of Applicants' disclosure and the known correlation of function and amino acid sequence structure render the claims in compliance with the written description requirement.

For these reasons, Applicants respectfully request reconsideration and withdrawal of the Section 112, 1st paragraph (written description) rejection.

Regarding the Section 112, 1st paragraph (enablement) rejection of Claim 35, Applicants have amended Claim 35, as suggested. Withdrawal of this rejection is respectfully requested.

Applicants believe that the foregoing is responsive to each of the points recited in the Office Action, and submit that the present application is in allowable form. Favorable consideration and passage to issue are earnestly solicited.

The Commissioner is authorized to charge Deposit Account No. 04-1928 (E. I. du Pont de Nemours and Company) for any requisite fees due or to credit any overpayment.

Applicants' undersigned may be reached at the below-listed numbers.

Respectfully submitted,



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Attachments: Amended drawings (2 sheets)
 Appendix A (1 page)
 Rogne et al. reference

APPENDIX A

Appendix A shows a comparison of the amino acid sequences of Lecithin:Cholesterol Acyltransferase from soy clone SID sl2.pk0015.e8:fis (SEQ ID NO:14) and the human Lecithin:Cholesterol Acyltransferase set forth in NCBI General Identifier No. 4557892. Residues that have been shown to play an important role in the catalytic activity of Lecithin:Cholesterol Acyltransferase are boxed, and segments predicted to be in alpha-helical conformation are underlined (Rogne et al.(1987) Biochem.Biophys.Res.Comm. 148,161-169). Furthermore, amino acids conserved among both sequences are indicated with an asterisk (*) on the top row. Dashes are used by the program to maximize alignment of the sequences.

**ISOLATION AND CHARACTERISATION OF A cDNA CLONE FOR
HUMAN LECITHIN:CHOLESTEROL ACYL TRANSFERASE AND ITS USE
TO ANALYSE THE GENES IN PATIENTS WITH LCAT DEFICIENCY AND
FISH EYE DISEASE**

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We have isolated cDNA clones coding for human lecithin:cholesterol acyl transferase (LCAT) from a liver-specific cDNA library by the use of oligonucleotide probes based on the protein sequence. The clones cover the sequence coding for the entire secreted LCAT, the 3' untranslated sequence and 12 amino acids of the signal peptide. The peptide sequence contains the conserved active site of serine lipases with a hydrophobic domain, flanked by a possible amphipatic α -helix. Only one gene for LCAT could be detected in genomic blots. We have used the cDNA as a probe to analyse the LCAT gene in patients suffering from LCAT deficiency and fish eye disease. No rearrangements or abnormal gene fragments were detected in these patients.

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Lecithin:cholesterol acyl transferase (LCAT, phosphatidyl-choline: sterol acyl transferase, EC 2.3.1.43) is an enzyme present in plasma that catalyses the transfer of fatty acid residues from phosphatidylcholine to cholesterol, thereby transforming an amphipatic cholesterol to a hydrophobic cholesteryl ester. The enzyme is secreted from the liver (1) and is found on the surface of high density lipoprotein (HDL) in a complex with apolipoproteins AI and D (2). Apolipoprotein AI, which is the

Acknowledgments:

LCAT, Lecithin:cholesterol acyl transferase; cDNA, complementary DNA; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; bp, basepair; RFLP, restriction fragment length polymorphism.

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major protein component of HDL, stimulates LCAT activity when synthetic liposomes are used as substrates (3). The function of apoD is unclear, it has been suggested to be a cholesteryl ester transfer protein, responsible for the transfer of LCAT-generated esters from HDL to very low density lipoprotein (VLDL) and low density lipoprotein (LDL) (4). The activity of LCAT seems important for the "reversed cholesterol transport" from peripheral tissues to the liver (5,6) because unesterified cholesterol can exchange freely between lipo-proteins and cell membranes whereas esterified cholesterol resides in the hydrophobic interior of the lipoprotein particles and can be exchanged only by the action of lipid transfer proteins (7,8).

In patients with LCAT deficiency accumulation of cholesterol in peripheral tissues can be observed, leading to corneal opacity, premature atherosclerosis and renal failure (9,10). Patients suffering from fish eye disease, a hereditary disease manifested by corneal opacities and dyslipoproteinaemia, also have abnormal LCA activity (11). Based on evidence of two different kinds of LCAT activity with different substrate specificities, it is possible that two slightly different genes for LCAT exist and that deficiency of one of them leads to fish eye disease (12,13).

As part of our effort to study the genes related to plasma lipid metabolism and thus might be involved in cardiovascular pathogenesis, we now report the cloning of a cDNA coding for LCAT and its use to analyse the LCAT genes of patients suffering from these diseases. These probes will be made available for research.

MATERIALS AND METHODS

Library screening. The cDNA library, made from adult human liver, was kindly provided by dr Derek Woods (14), and was plated and screened with labeled oligonucleotide probes as previously described (15). Since the oligonucleotides were 20 basepairs (bp) long, hybridisations were carried out at 45 °C.

DNA sequence analysis. Fragments of the cloned cDNAs were subcloned in the singlestranded M13 phage vectors mp8,9,18 or 19 (16) and both strands were sequenced by the dideoxy chain termination method. Sequence assembly and analysis was done on an Olivetti M24 personal computer using the Beckman Microgenie software and the BIONET databank and computing facility in Palo Alto, California. Protein analysis was carried out by the programs of the University of Wisconsin Genetic Computing Group and by the PC/GENE software package kindly provided by Genofit (Geneva).

Gene analysis. DNA was purified from blood samples after osmotic lysis of erythrocytes (17). Blood samples (20ml) were diluted in 4 volumes of RBC-lysis buffer (155mM NH₄Cl, 10mM KH₂CO₃ pH 7.4, 0.1 mM EDTA) and lysed for 15 min at 0°C. White blood cells were pelleted at 400g, washed in RBC-lysis buffer, resuspended in 0.9% NaCl, 25mM EDTA and lysed by the addition of SDS to 1%. The samples were digested overnight at 37° with 1.5mg pronase (Boehringer). DNA was diluted to 15ml and extracted thoroughly with phenol, phenol/chloroform and chloroform. Care was taken to mix the phases well by inverting tubes for 10 min during each extraction step. The DNA was precipitated by addition of 1/30 volume of 3M sodium acetate pH 5 and 1 volume isopropanol. The DNA was collected with a glass rod, rinsed in 70% ethanol and dissolved in 1ml TE (10mM pH 7.6, 0.1mM EDTA). This precipitation procedure facilitates the redissolving of DNA. DNA of high molecular weight (several hundred kb) can be obtained by this procedure. The yield is usually between 0.5 and 1 mg per 15-20 ml blood. Samples containing 5 µg DNA were cut with restriction enzymes and separated on 0.8% agarose gels and blotted onto nylon membranes (Biorad). The DNA was cross-

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membrane by UV-irradiation (800 μ W at 254nm for 20s; ref18). Probes were prepared by randomly primed synthesis on denatured cDNA inserts essentially as described (19); We add the homemade primers (ref 20, kindly provided by dr Tom Kistensen) to 25ng of the insert before boiling and add 5x buffer, nucleotides and enzyme separately afterwards. Labeling reactions were for 4-6 hours at room temperature (see also ref 21). Dried skimmed milk (Nestlé, 0.5%) and herring sperm DNA (100 μ g/ml) was used as blocking agents during hybridisations. Hybridisation conditions were as previously described (22). The patients have been described previously (11,23).

RESULTS AND DISCUSSION

cDNA cloning. Five hundred thousand clones from an amplification of the cDNA library were screened with the two oligonucleotide probes in combination (corresponding to amino acids 21 to 27 and 218 to 224 respectively; refs 24,25) and 14 positive clones were picked¹. Preliminary restriction analysis showed three different types of clones, having inserts of 0.8, 0.8 and 1kb, respectively. In one of the shorter clones one of the PstI sites flanking the cDNA had been destroyed during cloning, indicating that the clones represent three independent clones from the original library. The longest clone hybridised to both of the oligonucleotides whereas the shorter hybridised only to the more carboxy terminal probe. Restriction analysis and differential hybridisation to the oligonucleotide probes showed that the clones were overlapping, the longest covering the 5'end of the expected cDNA and the other covering the 3'end (fig 1).

The DNA sequence (fig 2) shows no start codon preceding the start of the secreted protein, thus indicating that we have cloned only part of the signal peptide at the amino terminal end. As might be expected the signal peptide consists mainly of hydrophobic amino acids, of which 6 are leucines. Following the stop codon at position 1287 there is only 23 bp of 3' untranslated sequence before the poly(dA) tail starts and the poly-adenylation signal is part of the Glu₄₁₆ and stop codons. The

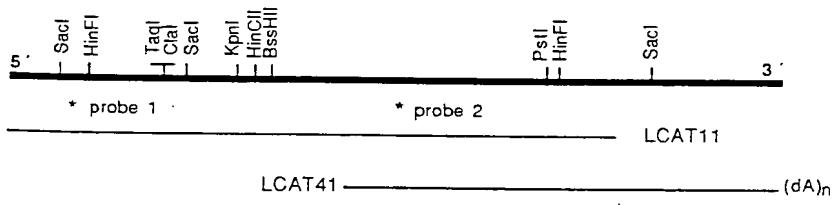


Figure 1. Restriction map of clones LCAT-11 and LCAT-41, showing the areas complementary to the oligonucleotide probes.

Prior to the work reported here we isolated and sequenced several clones which hybridised to oligonucleotides based on partial peptide sequences from LCAT (37). However none of these clones coded for LCAT, and we later found that this was due to single amino acid errors in the sequences.

Figure 2. The DNA sequence of the cDNA coding for human LCAT. Nucleotide number 1 corresponds to the first nucleotide following the G-linker of clone LCAT-1 and amino acid number 1 corresponds to the first amino acid of mature circulating LCAT. The active Serine is indicated by an asterisk. Segments predicted to be in α -helical conformation (cf text) is indicated by plus signs. Oligonucleotide probes indicated by solid lines. Predictions for β -sheet did not agree well with circular dichroism measurements (29) and are not shown.

biological significance of such an exceptionally compact 3' untranslated sequence is unclear.

The peptide sequence predicted from our cDNA clones is in full agreement with that recently found by protein sequencing (25)². Yang et al. also identified two disulfide bridges from Cys₅₀ to Cys₇₄ and Cys₃₁₃ to Cys₃₅₆ (25).

Homology to other lipases. A search for homology between LCAT and other lipases by the sensitive algorithm of Argos (26) revealed only small stretches of homology. One of these (fig 5) is of interest because it includes the active serine of other lipases (which are serine esterases). This, in addition to the involvement of serine in the catalysis by LCAT (27), strongly supports that LCAT has a similar active site and that Ser₁₈₁ is the active serine.

Protein secondary structure. By the aid of computer programs we tried to predict the secondary structure of the LCAT peptide. In particular we were interested in amphipatic α -helices, which would parallel the amphipatic α -helices found in apolipoproteins and are thought to be typical of proteins which function at a water/lipid interface. In spite of the inherent uncertainty of algorithms used to predict secondary structure (28), the fraction of LCAT predicted to be in the α -helical conformation is close to that found by circular dichroism analysis (25 and 24 % respectively, ref 29). Analysis by the algorithm of Garnier et al (28) indicated that a 21 amino acid segment from Gln₁₅₃ to Lys₁₇₃ may be α -helical. This is supported by the Chou and Fasman procedure (30) which give peak probabilities for the beginning of this segment being at the amino terminal end and the end of the segment being at the carboxy terminal end of an α -helix (results not shown). Figure 3 shows how this segment would have amphipatic properties, having hydrophobic residues on one side and hydrophilic on the other (omitting the first amino acid, Gln₁₅₃). In addition several shorter α -helical segments were suggested (indicated in fig 2), none of which had similar convincing amphipatic properties. The hydropathy plot (fig 4, ref 31) of the peptide shows several hydrophobic stretches, one of which (going from Val₁₇₅ to Leu₁₉₃, i.e. starting just after the possible amphipatic α -helix described above) surrounds the Ser₁₈₁ of the putative active site. This would enable the enzyme to act within the lipid surface of the lipoprotein, and the more hydrophobic nature of LCAT compared to the apolipoproteins (25) may be due to this requirement.

Gene analysis. We analysed the LCAT genes of 19 unrelated normal individuals, cut with one of 17 different restriction enzymes (and 6 individuals with 11

²During the progression of this work the sequence of another LCAT cDNA clone was published (24). This sequence agrees completely with ours, even at third base positions and untranslated sequence.

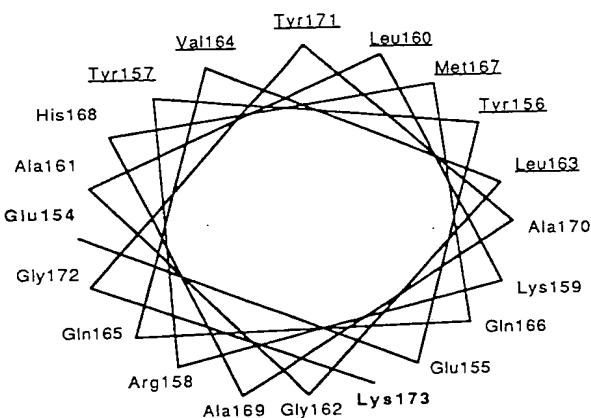


Figure 3. View along the axis of the proposed amphipathic α -helical segment from amino acid 153 to 173. Hydrophobic amino acids (i.e. residues having positive hydrophathy indices on the scale of Eisenberg; ref 32) are underlined.

other enzymes)³ in an attempt to detect restriction fragment length polymorphisms (RFLPs). In all cases the 38 (or 12) alleles were of the same size, precluding common RFLPs with these enzymes. We also analysed the genes of patients

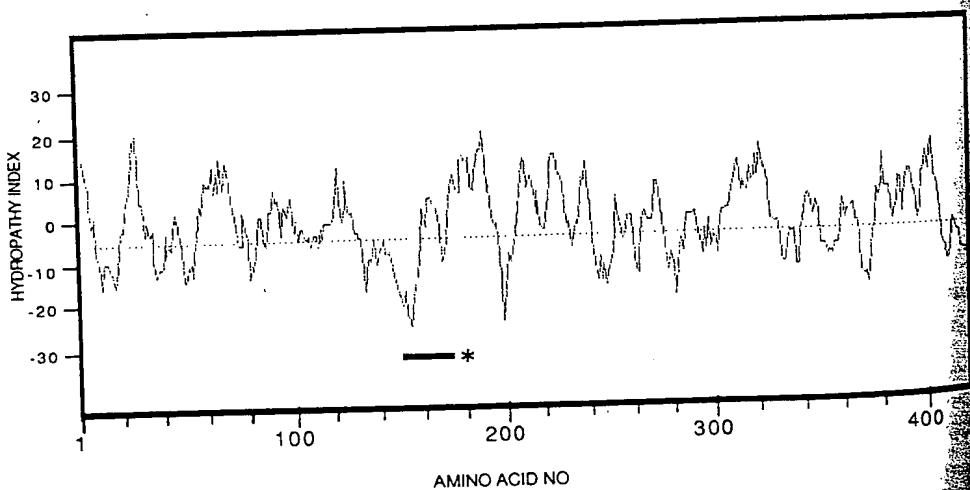


Figure 4. Hydropathy plot of LCAT. Average hydropathy of 9 amino acid segments were calculated for every position along the peptide. Positive values indicate segments more hydrophobic and negative values segments more hydrophilic than the average (31). The bar indicates the proposed amphipatic α -helix and the asterisk the active serine. The dotted line designates average hydropathy on the scale of Kyte and Doolittle (31).

³ The following enzymes were used: Apal, AluI, Accl, BamHI, BglII, BglIII, BssHII, Clal, DraI, EcoRI, EcoRV, HaeII, HaeIII, HinDIII, HinFI, HpaI, KpnI, MboI, MspI, PstI, Pvull, SacI, StuI, TaqI, XbaI, XhoI, XmnI. Those underlined were used to 19 samples.

Human LCAT	Phe Leu Ile Gly His Ser Leu Gly Cys Leu
Rat hepatic lipase (33)	His Leu Ile Gly Tyr Ser Leu Gly Ala His
Human lipoprotein lipase (34)	His Leu Leu Gly Tyr Ser Leu Gly Ala His
Porcine pancreatic lipase (35)	His Val Ile Gly His Ser Leu Gly His Ser
Rat lingual lipase (36)	His Tyr Val Gly His Ser Glu Gly Thr Thr

Figure 5. Homology of the segments surrounding the presumed active serine residue of LCAT and members of the lipase family.

ing from LCAT deficiency (23) and from fish eye disease (11). Although we compared the gene fragments generated by ten different restriction enzymes, several which had four bp recognition sequences and gave small fragments, no differences could be detected between patient genes and those of normal individuals (one example is shown in fig 6). The LCAT deficient patients studied here do have low levels of a defective LCAT protein (23). It is unlikely that deletions or insertions of more than 50 bp would evade detection by our detailed analysis. A point mutation giving a single amino acid exchange may induce conformational changes which result in altered enzyme activity, increased degradation rate or reduced secretion rate. To determine the exact gene defect an LCAT gene of one of these patients would have to be cloned and sequenced. Although with the sensitivity of our analysis the LCAT genes of the fish eye disease patients are indistinguishable from the normal genes, we cannot exclude that point mutations or minor rearrangements may have altered the gene product so as to give the characteristic LCAT activity described (12). Although we could not detect more than one gene for LCAT even at reduced stringency (washing in 150mM Na) it is still possible that another gene for an LCAT-like enzyme may exist, but that the homology between

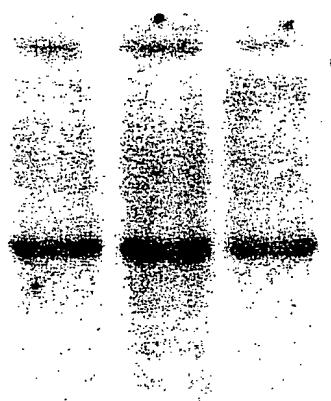


Figure 6. Southern blot of genomic DNA digested with Dral. Samples were (from left) an LCAT-deficient patient, a fish eye patient and a normal individual. The molecular weight of the fragments are 2.6 kb and more than 20 kb.

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these is less than 90% at the DNA level. We are at present investigating this possibility further. Our finding is supported by the detection of only one LCAT mRNA in liver (24).

Because LCAT is believed to play an important role in the removal of cholesterol from peripheral tissues it may be of importance to the development of cardiovascular disease. The isolation of a gene probe for LCAT enables the analysis of whether genetic variants may predispose to this disease. Although this work at present is hampered by the lack of RFLPs around the gene, the isolation of genomic clones for LCAT will hopefully provide polymorphic probes for genetic analysis.

ACKNOWLEDGEMENTS

We are indebted to professor G Kostner for his collaboration in our initial attempts to acquire LCAT peptide sequence, to dr Skarbøvik for assistance in collection of blood samples from LCAT patients and to Anne Engen, Hege Svendsen, Jorun Solheim and Reidun Jonassen for excellent technical assistance. This work was supported by the Norwegian Cancer Society and the Norwegian Council on Cardiovascular Diseases.

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